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RESEARCH ARTICLE

Levels of diversity and inbreeding in a clonal seed orchard for high-mountain ecotype of Scots Pine

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Abstract

Seed orchards are an important source of high-quality seeds for afforestation. Genetic composition of seed orchard crop was studied in a clonal seed orchard situated in Borovets, Rila Mts, which was designed for producing seed for high-mountain afforestations. Diversity in the seed orchard was comparable with the results of other similar studies. Both multilocus and single-locus outcrossing rates were relatively low – 0.867 and 0.782, respectively. Such low levels of outcrossing are not unprecedented in conifers, but the results indicate some degree of selfing in the seed orchard. The possible causes and factors influencing genetic aspects of the reproductive process in the seed orchard are discussed.

Keywords
Pinus sylvestris, outcrossing rate, seed orchard, hybrids

Introduction

The traditional concept of ecotypes is more than 100-year old (Turesson, 1922). The interpretation of ecotypes by different researchers and for different groups of organisms varies considerably. In their recent review Stronen et al. (2022) summarize that defining ecotypes is most frequently based on habitat specificity, rather than on genetic background. In forest trees the concept gained popularity and has been extensively applied in gene conservation and breeding zones' designation. Recently, much

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more emphasis was given to the local adaptation, represented either by ecotype or cline variation and shaped by extensive gene flow (Savolainen et al., 2007).

Usually three ecotypes of Scots Pine (*Pinus sylvestris* L.) are distinguished in Bulgaria, depending on the altitude. The low-mountain one is represented by small isolated populations situated at 800-900 m a.s.l. The middle-mountain ecotype (roughly between 1000 and 1800 m a.s.l.) represents the optimum of the species distribution and the populations here are characterized by the best phenotype quality. Finally, the high-mountain ecotype occurs in the zone of alpine timber line (2000-2100 m a.s.l.) and is represented by isolated stands mostly in Rila Mts. (Dobrinov et al., 1982). Modern concepts of seed supply for afforestation consider delineation of breeding zones, presuming that the local populations should be best adapted to the respective environmental conditions (Zobel, Talbert, 1984; Westfall, Conkle, 1992; Crowe, Parker, 2011; Yu et al., 2022). In the case of Bulgarian Scots Pine, breeding zones were delineated vertically, based on ecotypes defined by altitude.

Numerous seed orchards were established during the last 50-60 years in Bulgaria (Alexandrov, Dobrev, 2011). Of the many clonal seed orchards for Scots Pine, three were especially designed following the ecotype variation – for the low-mountain ecotype situated near Sofia (800 m a.s.l.), for the middle-mountain ecotype situated near Samokov (800 m a.s.l.) and for the high-mountain ecotype in the region of Borovets ski resort (1300 m a.s.l.). Each of the three seed orchards comprises clones, representing plus trees selected in the natural populations of the respective ecotype with some additional clones (Dobrinov, Gagov, 1980). The seed orchard in Borovets was designed for providing seeds for afforestation at higher altitude, in the zone of the alpine timber line. It was proven and it is believed that the mating among clones included in the orchard will yield good-quality seeds with progeny able to adapt to the severe high-mountain conditions.

There are numerous indicators to assess the effectiveness of a seed orchard – fertility, flowering synchrony, high genetic diversity in the seed crop, low level of background pollen contamination, just to mention some of them. All these parameters had been studied in detail in the recent few decades in many tree species, including Scots Pine (Muona, Harju, 1989; El-Kassaby et al., 1989; Kang, Lindgren, 1999; Prescher et al., 2007; see Kang, Bilir, 2021, for review). Many of the above-mentioned parameters still remain to be studied in the seed orchards of conifers in Bulgaria.

Therefore, the objective of the present work was to describe the genetic composition, levels of diversity and inbreeding in the seed crop of Borovets clonal seed orchard.

Material and methods

The clonal seed orchard is situated in the locality called Byala polyana, (42°17.70' N, 23°38.35 E, 1290 m a.s.l.). It was established in the period 1980-1985 by grafting and encompasses clones of 30 plus trees from four natural populations situated

within the range 1600-2100 m altitude (Dobrinov, Gagov, 1980). The provenances represented in the seed orchard are: Panichishte – six clones, Saragyol – ten clones, Yundola – six clones, and Yakoruda – three clones. The provenances Saragyol and Yakoruda represent the high-mountain ecotype and some individuals of these provenances represent spontaneous hybrids between *P. sylvestris* and *P. mugo*. By the time of the orchard establishment, each clone was represented by 15-25 ramets, and our field observation at the moment of the study revealed an average survival ratio of about 60 %. A scheme with the distribution of ramets of the clones at the moment of the seed orchard's establishment is presented in the supplementary material (S1). Cones were collected in 2015 from individuals belonging to 18 clones. Seeds were extracted from the cones manually and were kept in a refrigerator until the analysis. They were germinated on moist filter paper and parallel analysis of seven endosperms and the respective embryos per individual was performed by employing allozyme gene markers. Five enzyme system codings for eight interpretable gene loci were analyzed, namely Glutamate-oxaloacetate transaminase (loci GotA, GotB and Got C), Menadione reductase (locus Mnr), Glutamate dehydrogenase (locus Gdh), Malate dehydrogenase (loci Mdh-A and Mdh-B) and Shikimate dehydrogenase (locus Skdh-B). Enzyme extraction, electrophoretic and staining procedures, locus and allele designation followed Hertel et al. (1999).

Genotypes of the clones (parental individuals) and the respective embryos were scored from electrophoregrams. The use of seven endosperms allowed reliable identification of parental genotypes (see Zhelev et al., 2010). Allele frequencies in the pollen cloud and ovules, outcrossing rates and inbreeding coefficients were calculated using the mixed mating model of Ritland (2002) with the software MLTR for Windows, version 2.4. (Ritland, 2002). Allele frequencies in the next generation were calculated based on diploid embryo genotypes, with standard errors obtained as $SE_{(pi)}=[p_i(1-p_i)/2N]^{1/2}$, where p_i is the frequency of the allele of interest and N is the number of embryos.

Results and discussion

Diversity

The studied loci were polymorphic in all variants with only two exceptions – Mdh-A in the pollen cloud and Got-A in the ovules. In all remaining cases the loci studied were polymorphic. Allele frequencies had similar values in the all three cases studied (Table 1). Diversity parameters based on the allele frequencies (Table 2) were relatively high. Expected heterozygosity based on average allele frequencies of the pollen cloud and ovules ranged from 0.071 for locus Got-A to 0.448 for locus Skdh-B, with a mean value of 0.286. Observed heterozygosity in the next generation of the orchard represented by the embryos of the seeds studied ranged from 0.064 (locus Got-A) to 0.392 (locus Mdh-D), with a mean value of 0.281. In most cases, with the exception of locus Skdh-B, the differences were of small magnitude.

Effective allele number, which is used also as a measure of diversity (Crow, Kimura, 1970; Gregorius, 1987, 1991) ranged from 1.066 (locus Got-A) to 1.646 (locus Gdh), with a mean value of 1.4.

The results indicate that the diversity parameters established in the seed orchard were of magnitude traditionally reported for the populations of coniferous species and particularly, for seed orchards of Scots Pine (Szmidt, 1987; Muona, Harju, 1989; Burczyk, 1991; Hamrick et al., 1992; Dzaliuk, Burczyk, 2002).

Table 1. Estimated allele frequencies (standard errors in parentheses) in the pollen cloud and in the ovules, and in the next generation (embryos) in the seed orchard

Locus	Allele	Pollen	Ovules	Embryos
Got-A	1	0.077 (0.051)	0.000 (0.000)	0.032 (0.012)
	3	0.923 (0.051)	1.000 (0.000)	0.968 (0.012)
Got-B	4	0.226 (0.102)	0.194 (0.054)	0.222 (0.028)
	5	0.774 (0.102)	0.806 (0.054)	0.778 (0.028)
Got-C	2	0.236 (0.121)	0.194 (0.059)	0.285 (0.038)
	4	0.764 (0.121)	0.806 (0.059)	0.715 (0.038)
Mnr	2	0.688 (0.110)	0.833 (0.051)	0.732 (0.034)
	3	0.312 (0.110)	0.167 (0.051)	0.268 (0.034)
Gdh	2	0.271 (0.103)	0.194 (0.076)	0.225 (0.029)
	3	0.729 (0.103)	0.806 (0.076)	0.775 (0.029)
Mdh-A	1	0.000 (0.000)	0.028 (0.022)	0.019 (0.009)
	3	1.000 (0.000)	0.972 (0.022)	0.981 (0.009)
Mdh-D	1	0.361 (0.094)	0.167 (0.054)	0.224 (0.028)
	2	0.639 (0.094)	0.833 (0.054)	0.776 (0.028)
Skdh-B	1	0.278 (0.104)	0.444 (0.093)	0.375 (0.035)
	2	0.722 (0.104)	0.556 (0.093)	0.625 (0.035)

Outcrossing

The multilocus estimate of outcrossing rate (t_m) was 0.867 (±0.061). An approximate interpretation of this result is that about 87 % of the seeds of the orchard result from outcrossing and some 13 % originate from selfing, which is relatively low proportion. Mean single-locus estimate of the outcrossing rate (t_s) was lower – 0.764 (±0.069). The difference between the multilocus and single-locus estimates indicates that there are factors affecting the pollination and fertilization process other than selfing, like gametic disequilibrium, and violation of some assumptions underlying the model. An unequal contribution of the different clones to progeny could also bias the results. However, precise phenological observations are necessary to evaluate the effective number of clones and their synchrony in the flowering process. The

seed orchard is well isolated and background pollination, if any, is expected to be minimal. Outcrossing rates obtained in the present study are substantially lower than these reported in other studies of Scots Pine seed orchards, which are usually from 0.91 to 0.99 for t_m (Koski, Muona, 1986; Muona, Harju, 1989; El-Kassaby et al., 1989; Burczyk, 1991) and even higher for t_s (e.g., 1.011, Burczyk, 1991). However, similar relatively low levels of outcrossing are not unprecedented. For example, Paule, Mrazikova (1990) found similar levels of outcrossing (t_m=0.79, t_s=0.63) in a Scots Pine clonal seed orchard in Slovakia. Low lelevs of outcrossing were reported also in many other studies of Pine species, like *P. radiata* (t_m= 0.75, Rogers, 2002), *P. leucodermis* (t_m=0.76-0.84, Morgante et al., 1991), *P. sibirica* (t_m= 0.85, Politov, Krutovsky, 1990), *P. merkusii* (t_m=0.17-0.84, Changtragoon, Finkeldey, 1995).

Table 2. Diversity parameters

	He	**Ho**	**n_e**
Got-A	0.071	0.064	1.066
Got-B	0.331	0.352	1.528
Got-C	0.337	0.375	1.688
Mnr	0.354	0.345	1.527
Gdh	0.354	0.372	1.646
Mdh-A	0.027	0.037	1.535
Mdh-D	0.370	0.392	1.039
Skdh-B	0.448	0.312	1.533
Mean	0.286	0.281	1.400[1)]

Legend: He – expected heterozygosity based on the average allele frequencies in the pollen cloud and in ovules; Ho - observed heterozygosity in the progeny (embryos); n_e – effective allele number, based on the allele frequencies in the embryos.
[1)] harmonic mean

Inbreeding coefficient (*F*) was negative but not significantly different from zero (-0.008). Ideally, in an equilibrium with the population the relationship between outcrossing rate and inbreeding coefficient should be F=(1-t)/(1+t), which in the present case should be 0.07. In fact, it is substantially lower, which indicates that the factors and processes underlying the low level of outcrossing rate are much more complex than the selfing itself (Hedrick et al., 1999).

The correlation parameters r_t and r_p had similar and relatively low values, indicating differences among the clones and also, in the case of r_p, that the progeny of a given clone results from pollination by multiple paternal trees. However, the inferences must be cautious given the large standard errors (Table 3).

Generally, for the seed orchard we could state that although the outcrossing rates are lower than the ones obtained in other studies on *P. sylvestris*, still 87 % of

Table 3. Outcrossing rates, inbreeding and the associated correlations

Parameter	Newton-Raphson method estimate (s.e.)
t_m	0.867 (0.061)
t_s	0.764 (0.069)
Difference tm-ts	0.103 (0.036)
F	-0.008 (0.106)
r_t	0.091 (0.297)
r_p	0.162 (0.074)

Legend: t_m – outcrossing rate, multilocus estimate; t_s – outcrossing rate, average single-locus estimate; F – inbreeding coefficient (mean of all studied clones); r_t – correlation of t-estimate r_p – correlation of outcrossed paternity

the seed orchard crop result from cross pollination. It is worth noting that these results concern the crop of a particular year and there could be differences in the studied parameters in different years caused mostly by environmental conditions (Miguel et al. 2002, Whitehead et al. 2018). Phenological observations could bring valuable data about the flowering synchrony among clones, which is of particular importance considering that the orchard comprises clones belonging to "pure" *Pinus sylvestris* and clones belonging to its hybrids with *P. mugo*. Also, progeny testing will be necessary for obtaining information about the performance of different clones' progeny. This will allow their ranking (backward selection) and together with the other information about flowering synchrony and fertility will help selection of best clones for next-generation seed orchards.

Conclusions

The results allow some inferences about the future use and management of the seed orchard:

The studied seed orchard is characterized by relatively high genetic diversity, as compared to other studies on Scots pine or other Pine species.

Outcrossing rates are relatively low but not unique, since similarly low levels of outcrossing were reported for many Pine species, including Scots Pine.

Although the outcrossing rates were low, seed orchard crop still can harbor high gene diversity and can be a valuable source of reproductive material leading to genetic improvement.

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